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ACTIVATED SLUDGE AS AN ECOLOGICAL SYSTEM

George W. Malaney

Vanderbilt University
Nashville, Tennessee

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1. List any publications pending or published and not previously reported.
2. List and describe any foreign travel undertaken during the above period.
3. Describe accomplishments since last summary progress report. Specify and describe the significance of any changes in the direction taken by the project during the above period.

1. J. J. Cibulka and G. W. Malaney, "Experimental Conditions in the Study of the Physiological Ecology of Activated Sludge." Paper presented at Purdue Industrial Waste Conference, 1967. (Copies Enclosed)

2. None

3. The progress report which follows is presented under the following headings:
   A. Isolation and Propagation of Bacteria from Activated Sludge.
   B. Identification of Bacterial Strains Isolated from Activated Sludge.
   C. Warburg Studies (Continued)
   D. Enumeration of Aerobic Bacteria in Activated Sludge
   E. Analysis of Primary Effluent for Content of Saturated Monocarboxylic Acids.

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A. Isolation and Propagation of Bacteria from Activated Sludge

Step 1.

The medium employed for isolation of activated sludge microorganisms must satisfy three fundamental criteria; (1) contain little enrichment in addition to those natural nutrients occurring in the aeration tank, (2) stimulate colony growth in a period of a few days, (3) should exhibit colonies of distinctive morphological characteristics to enhance selection of individual species. In studies previously reported, the medium optimizing the above criteria was one containing 2.3% nutrient agar (by weight) in solution in a diluent consisting of 50% distilled water, 25% settled sewage, and 25% activated sludge mixed liquor (ASML). This diluent is referred to as SS-ASML. The ASML is prepared for use in the diluent by blending aeration tank liquor for 135 sec, followed by centrifugation for 5 min at 1600 rpm. The diluent and agar were mixed and brought to a boil to dissolve the agar. The solution was autoclaved at 121°C for 20 minutes in cotton-stoppered flasks and subsequently poured into sterile petri plates. This medium was named SS-ASML nutrient agar (NA-SS-ASML). Organisms were inoculated by the surface-plate technique on the surface of the hardened agar. Activated sludge mixed liquor (ASML) from the Franklin plant was thoroughly shaken to insure a uniform mixture of organisms. One hundred ml of the mixed liquor was blended for 135 sec and dilutions to 10^{-6} were made using sterile BOD dilution water prepared as in Standard Methods for the Examination of Water and Wastewater. Volumes ranging from 0.1 ml to 1.0 ml of 10^{-6} dilution were pipetted onto the hardened agar surface and spread by tilting the plate. After 6 days incubation at 25°C, bacterial plate counts on the SS-ASML nutrient agar medium averaged 1.3×10^{8} organisms/ml of ASML. Distinctive characteristics of colonies on this medium included variations in size, texture, form, margin, surface, elevation, optical character and color.

The addition of nutrient agar to the isolation medium was necessary since SS-ASML diluent alone solidified with agar resulted in meager and non-distinctive colonial growth after 9 days incubation at 25°C. The 50% concentration of water in SS-ASML was chosen since both greater and lesser amounts of water resulted in a reduction of colony numbers. The 1:1 ratio of SS to ASML was also optimum for development of distinctive colonial colors which greatly aided initial selection of bacterial types. The 25°C incubation temperature producted distinctive colonies on this medium after 6 days growth while incubation at 20°C resulted in colonies somewhat less well developed in the same time interval. Therefore, all organismal characteristics of chosen species were established on the basis of 6-day growth on NA-SS-ASML at an incubation temperature of 25°C.

Washing of the activated sludge prior to blending was discontinued after plate studies of washed and unwashed sludges showed that the washing procedure caused a selective loss of organisms. Also, parallel Warburg oxidation studies employing washed and unwashed sludges indicated a difference in oxidation pattern. This point was discussed in the Purdue paper.

Step 2.
Distinctive colonies on Step 1 plates were streaked onto the surface of hardened NA-SS-ASML. After 24-hour incubation at 25°C, growth on the Step 2 plates was spread using a sterile glass rod. Following an additional 72-hour incubation at 25°C, the plates were carefully examined under 10X magnification. Those plates which showed the presence of a single colonial type were considered pure cultures and were used for propagation in Step 3.

Step 3.
Propagation was carried out on medium made up of 2% Bacto agar in SS-ASML. This unenriched agar (SS-ASML agar) was used for maintaining stock cultures

and for propagation of bacteria just prior to use in the Warburg studies.

After a period of prolonged growth (3-5 weeks) on SS-ASML agar, the pure cultures were streaked on NA-SS-ASML to determine if the chosen types maintained their morphological integrity. Examination after 6 days incubation indicated that all cultures exhibited the same distinctive characteristics present at the time of the original Step 2 isolation. This would seem to indicate that variations due to mutation and selection were inconsequential in this study. However, this is not meant to exclude the possibility of variation, which deserved detailed study.

Step 4.
Comparative Warburg oxidation investigations employing the same organism grown on NA-SS-ASML and SS-ASML agar (unenriched) just prior to addition to the Warburg flasks resulted in dissimilar 24-hour oxygen uptake curves indicating that the organisms grown in the two media did not oxidize substrates at the same rate. Since simulation of natural environmental substrate metabolization is imperative in this study, it was decided to use only cultures grown on SS-ASML agar in the Warburg studies. This decision became even more valid when oxidation curves of whole sludge (i.e., ASML) showed a pattern more similar to that of the cultures grown on SS-ASML agar than those organisms grown on NA-SS-ASML.

In order to accumulate sufficient mass of bacteria on SS-ASML agar in a limited time just prior to use in a Warburg run, the following method of propagation was devised:

(a) Each bacterium was transferred to fresh SS-ASML agar plates every 7-10 days.

(b) Forty-eight hours prior to the Warburg run, the bacterium was streaked on the surface of hardened SS-ASML agar plates and spread with a loop.

Four to ten plates were streaked, depending on growth rate of the specific bacterium.

(c) After 24 hours incubation at 25°C, the bacteria on the surface of each plate were fed by adding 2 drops of sterile feed. The feed was brought in contact with the organisms by cross-streaking with a sterile loop. This spreading and feeding resulted in a rapid increase in bacterial mass during the ensuing 24-hour period.

The feed preparation was made as follows:

1. ASML sludge solids were allowed to settle by standing and the resulting liquor decanted off.
2. The liquor was blended for 15 sec.
3. The blended liquor was filtered in a Buchner funnel through Whatman #589 white ribbon filter paper followed by a second filtration through Whatman #589 blue ribbon filter paper.
4. The filtrate was again filtered, this time through a sterile millipore filter (HA) and collected in several sterile flasks.
5. The sterile filtrate containing dissolved nutrients was stored in the refrigerator until use.
6. Each flask of sterile feed was used only once in order to avoid contamination.

(d) After 48 hours total incubation time, the bacteria were harvested from the agar surface by adding 10 ml of phosphate buffer solution and emulsifying with a sterile glass rod.

(e) The bacterial-buffer suspension thus produced on each plate was pipetted into a sterile bottle containing beads.

(f) The bottle was vigorously shaken for 15 sec to break up the bacterial agglomerates.

(g) The bacterial concentration (w/v) in the bottle was determined by the millipore filter technique, followed by dilution to 80 mg/l with buffer solution.

(h) The standard volume of this bacterial suspension added to each Warburg flask was 10 ml.

The amount of bacterial mass produced using the Step 4 technique varied with each bacterial type. Four plates were sufficient for the rapid developers and 10 plates for the slower ones. The average yield by this method was 1.8 mg per plate (dry weight).

Colonial Types

Initially 12 distinctive colonial types were recognized and picked from the Step 1 NA-SS-ASML plates for Step 2 isolation and propagation. Repeated Step 1 isolations led to the conclusion that only 10 of the original 12 types were distinctly different. Also, while some colonial types were distinctive their occurrence was sporadic and in such small numbers as to indicate a relatively unimportant role in aeration tank biological activity.

Six colonial types were selected for final Step 2 isolation and use in the Warburg studies. These 6 types occurred on all Step 1 isolation plates and represented a significant proportion of the total colonies present. Accumulatively, the 6 types represent 80% of bacterial colonies developed in Step 1. No attempt was made to relate frequency of occurrence by mass.

The six colonial types chosen for further study are coded and characterized as follows:

W-1 Round, elevated, glistening, translucent, buff-white color with brownish or greenish cast, smooth margin, 3 to 6 mm diameter. Average ratio - 26%.

WP-1 Round, elevated with peak, white, dull, irregular to smooth margin, 3 to 4 mm diameter. Average ratio - 10%.

YG-1 Round, elevated, yellow-green color, smooth margin, 4 mm diameter. Average ratio - 5%.

O-1 Round, elevated, glistening, deep orange color, smooth margin, 1 mm diameter. Average ratio - 4%.

Y-1 Round, elevated (may have peak), bright to faint yellow color, smooth margin, 3 mm diameter. Average ratio - 6%.

WS-1 Round, punctiform, shiny, pure milk white, smooth margin 1 to 3 mm diameter. Average ratio - 29%.

Characteristics and ratios of the six types were based on growth of organisms from dilution of blended whole sludge after 6-day incubation at 25°C on SS-ASML nutrient agar.

The six bacterial types exhibit different rates of growth on SS-ASML agar in 48 hours at 25°C. Average dry net yield per plate for each organism is as follows:

YG-1	2.6 mg
WS-1	2.5 mg
Y-1	1.7 mg
W-1	1.7 mg
O-1	1.4 mg
WP-1	1.0 mg

B. Identification of Bacterial Strains Isolated from Activated Sludge.

As a preliminary to more intensive study, one culture of each of the six colonial types described above was isolated and purified by standard bacteriological techniques. Identification of these organisms was begun using the conventional tinctorial, cultural and biochemical tests, including:

1. Gram stain (Hucker modification, on NA growth after 24- and 96-hr incubation), for gram reaction, size, shape and arrangement.
2. Flagella stain (Leifson method) for type of flagellation.
3. Microscopic examination of wet mount, for shape, arrangement, motility.
4. Optimum growth temperature.
5. Type of growth on NA plates.
6. Type of growth in nutrient broth tubes.
7. Oxygen growth requirements.
8. Type of colonial growth on selective and differential culture media, such as: Pseudomonas agar-F, Pseudomonas agar-P, nitrogen-free agar, NA-SS-ASML, EMB agar, MacConkey agar, Kligler Iron agar, Violet Red Bile agar, pH 4.5 agar and TTC Overlay agar.
9. Biochemical reactions including hydrolysis of agar in 30 days, fermentation of glucose and lactose, gelatin liquefaction, oxidase production, and catalase production.

In parallel with the unknown cultures, the same tests were run on pure cultures of known bacteria obtained from the American Type Culture Collection. These known cultures were predominantly gram-negative species which have previously been identified as members of the microflora of activated sludge or which migh reasonably be expected to be present. These ATCC cultures were: Nima polymorpha, Achromobacter xerosis, Arthrobacter globiformis, Alcaligenes faecalis, Azotomonas insolita, Zymomonas mobilis, Aeromonas hyd phila, Agro-

bacterium tumefaciens, Acetobacter aceti, Rhizobium leguminosarum, Corynebacterium terrigena, Thiobacillus novellus, Flavobacterium aquatile, E. coli, Proteus vulgaris, Pseudomonas aeruginosa, Erwinia amylovora, Pseudomonas fragi, Salmonella paratyphi, Serratia marcescens, Aerobacter cloacae, Bacillus subtilis, Staphylococcus aureus.

Identification of the organisms will, if possible, be made by the method of Sneath (J. Gen. Microbiol. 17, 201, 1957). This is a grouping according to over-all similarity or natural resemblance, this quality being measured by mathematical and statistical techniques. This over-all similarity (S value) is calculated from observed characteristics.

C. Warburg Studies (Continuation)

The respirometer studies of the aerobic oxidation of the family of saturated monocarboxylic acids from C1 to C18 by whole sludge and by pure cultures of bacteria isolated from the sludge has resulted in a large volume of data which, unfortunately, is not presently in a form suitable for presentation.

D. Enumeration of Aerobic Bacteria in Activated Sludge.

One phase of the research involved determination of the proportion of the total bacterial population capable of metabolizing the individual monocarboxylic acids. These proportions had to be based upon reliable methods for measuring bacterial densities. It is well known that many experimental conditions influence the estimation of bacterial populations by the plating method. A study was made of the effect of variations in methodology upon the plate count of bacteria in activated sludge solids and aeration tank mixed liquor.

Sterilization of Waring blender jar. If a reasonably accurate bacterial count was to be obtained, it was essential that the aggregates of bacteria in the

sludge floc be effectively dispersed. Blending was the method of choice.

The method of sterilizing the blender vessel became a matter of concern. It was soon learned that several autoclave-sterilizations usually produced cracks in the blender jar with subsequent leakage. Sterilization with organic solvents was adopted. Sufficient acetone to cover the blades was added, the lid was replaced, and the blender was operated for a few seconds. The blender jar was then removed and manipulated so as to bring the acetone into contact with all inner surface. The blender was allowed to stand for 30 min. The rinsing was repeated and the acetone discarded. The jar was heated gently to drive off the acetone.

Sterility of the blender was testing by rinsing the inner surfaces with heat-steriled tap water and plating 1-ml portions of the rinse water on nutrient agar. Repeated tests gave no evidence of bacterial survival following acetone treatment.

Effect of blending time upon bacterial count. One hundred ml of aeration tank mixed liquor (ASML) was poured into a sterile Waring blender jar and blended for various times. At the end of each time interval, dilution of the blended suspension was made in sterilized tap water blanks and pour plates on nutrient agar were prepared by standard pour plate count (SPC) method. Incubation temperature was 28°C. Representative results are given in Table 1.

The results indicate maximum dispersion of floc and, therefore, maximum bacterial count was obtained by blending in the neighborhood of 2 min. A blending time of 135 sec was adopted as standard procedure.

Effect of incubation period upon bacterial count. A 100-ml sample of Franklin ASML was blended and various volumes plated out on nutrient agar using the SPC technique. The number of colonies on the plates were counted at various time

intervals. Results are given in Table 2.

It is obvious that the incubation time required to obtain a stable bacterial count would be prohibitively long, therefore, it was decided to incubate from 2 to 6 days, depending upon circumstances.

Effect of primary effluent in culture medium upon bacterial count. It was possible that nutrient agar prepared with distilled water might be lacking in certain essential ions or growth factors which, in the treatment plant, enter the aeration tank in the primary effluent. Bacterial counts were set up on blended Franklin ASML using the following culture media:

1. Nutrient agar (NA); 23 g per liter in distilled water.
2. NA; 23 g per liter in undiluted, unfiltered primary effluent (PE) from Franklin plant.
3. NA; 23 g per liter in undiluted primary effluent which had been filtered through cotton.

Representative results given in Table 3 indicate no advantage in terms of bacterial productivity in the addition of primary effluent to the culture medium.

Effect of aeration tank mixed liquor in culture medium upon bacterial count. It was possible that the liquid phase in the aeration tank migh contain growth factors or ions essential to the growth of the sludge bacteria. Bacterial counts were set up on Franklin ASML using the following culture media:

1. NA, in distilled water.
2. NA, in undiluted, unfiltered supernatant liquid from Franklin ASML which had been allowed to settle.
3. NA, in undiluted supernatant liquid from settled ASML which had been filtered through cotton.

The results (Table 4) are contradictory, indicating that in experiment no. 1 unfiltered ASML produced a higher count, while in experiment no. 2 produced a lower count

Effect of enriched culture media upon bacterial count. Trypticase soy agar (TSA) is said to be superior to infusion agar in nutrient quality and is recommended for the isolation of fastidious bacterial species. Diagnostic Sensitivity Test Agar (DSTA) is a highly nutritious medium which is useful as blood agar base to support the growth of delicate pathogens, as well as less demanding organisms.

Bacterial counts were prepared in the usual way with the following culture media:

1. NA, in tap water.
2. TSA, in tap water.
3. DSTA, in tap water.

Results are shown in Table 5. The two enriched media failed to support growth as well as nutrient agar.

Effect of diluted mixture of primary effluent (settled sewage) and aeration tank mixed liquor on bacterial growth. In the search for a culture medium promoting greater bacterial productivity, the following combination was made up for use as diluent. ASML was blended 135 sec, centrifuged 5 min and then allowed to settle. Seventy-five ml of the supernatant was added to a mixture of 75 ml SS and 150 ml distilled water. This diluent is coded SS-ASML.

Pour plates were prepared in the usual way with the following culture media:

1. NA, in tap water
2. NA, in SS-ASML
3. TSA, in tap water
4. TSA, in SS-ASML
5. DSTA, in tap water
6. DSTA, in SS-ASML

The results of two runs are given in Table 6. It is clear that the complex

diluent did not contribute to a higher bacterial count.

Bacterial Count of Activated Sludge in Presence of Acetic Acid as sole carbon source. In order to determine that proportion of the total bacterial population of activated sludge mixed liquor capable of using a fatty acid as only source of carbon, plate counts were set up in the usual fashion on the following culture media:

1. NA, in distilled water
2. Mineral salts agar (MSA), 7.0 g K_2HPO_4; 5.0 g KH_2PO_4; 1.0 g $(NH_4)_2SO_4$; 1.0 g KNO_3; 1.0 g $MgSO_4 \cdot 7H_2O$; 20.0 g agar per liter (in distilled water), pH 6.8.
3. Mineral salts-acetate agar (MSAA), prepared by adding filter-sterilized solution of sodium acetate to sterile MSA to give concentration of 500 mg per liter.

Representative results are shown in Table 7.

The experiment was repeated with the exception that tap water was used as diluent instead of distilled water (Table 8). It is apparent that the type of diluent had little influence on the proportion of acetate-utilizers at the two time intervals.

Proportion of acetate-utilizer when BOD dilution water was used as Dilution Blanks. Bacterial counts were set up using the same media as in the previous experiment, but the dilution blanks in this experiment were BOD dilution water (pH 7.1 - 7.2).

Results showed (Table 9) that, under the new experimental conditions, the proportion of acetate-utilizers at 4 days was essentially the same as before, but is much lower at 2 days. The difference resulted largely from increased bacterial growth on NA suggesting that many of the organisms were temporarily inhibited by exposure to the unadjusted tap water. The values for MSA represent growth of autotrophic bacteria. It must be presumed that these bacteria will also grow in the presence of fatty acid salt
~~also grow in the presence of fatty acid salt~~ therefore the MSA value must be

subtracted from MSAA count in calculation of ratio of acetate utilizers.

Influence of Beef Extract upon Growth of Acetate Utilizers. It was possible that the synthetic MSAA might lack some essential inorganic ion or growth factor. Beef extract is an excellent source of such nutrients. A catalytic amount of beef extract was added to determine if it increased the growth of acetate-utilizing bacteria. The following culture media was used:

1. NA
2. MSA, as before
3. MSAA, as before
4. MSA, to which was added 20 mg/l beef extract
5. MSAA, to which was added 20 mg/l beef extract

Representative results are given in Table 10. It is apparent that beef extract had little or no effect in increasing the count of acetate-utilizers, therefore, there is no point in adding this ingredient.

Effect of Variation in the Phosphate Buffer System used in Synthetic Culture Media. Since an excess of phosphate ion has been shown to be inhibitory to the growth of certain bacteria, MSA medium was modified by lowering the phosphate concentration to ca. M/25. The proportions of the two phosphate salts were also changed. The following media were used in the plate counts:

1. NA
2. MSA, as before
3. MSAA, as before
4. Modified MSA, as before except 4.0 g K_2HPO_4; 2.0 g KH_2PO_4 per liter, pH 7.0.
5. Modified MSAA, sterile sodium acetate solution added to modified MSA to give concentration of 500 mg/l.

Results are shown in Table 11. The modified synthetic medium more than doubled the count of acetate-utilizing bacteria. At the same time there was a reduction

in the number of colonies developing on the synthetic medium in the absence of carbon source.

Effect of Method of Sterilization of Culture Medium upon Productivity of Mineral Salts - Fatty Acid Agar. In previous experiments, sterilization of the synthetic medium had been accomplished by adding membrane-filter-sterilized acetate solution to autoclave-sterilized MSA. Unfortunately, the salts of the higher fatty acids form colloidal dispersions and some of dispersed phase might be lost during filter-sterilization. The feasibility of sterilizing the complete mineral salts-fatty acid agar in the autoclave was investigated using culture media prepared as follows:

1. MSAA, with sodium acetate solution filter-sterilized, then added to autoclave sterilized modified MSA (see previous experiment) to give concentration of 300 mg/liter acetic acid.
2. MSAA, with sodium acetate (300 mg/l) in modified MSA, sterilized in autoclave.
3. MSPA, as in 1 but using sodium propionate.
4. MSPA, as in 2 but using sodium propionate.

Results are given in Table 12. It is obvious that steam pressure sterilization reduced the productivity of the culture medium. The underlying cause was probably loss of part of the fatty acid by volatilization. As a consequence of this difficulty and the poor solubility of higher molecular weight fatty acid salts, the following standard procedure was adopted. The correct weight of unsterilized solid salt was added to heat-sterilized MSA. Possible contamination was checked by pouring a control plate of each flask of MSA-fatty acid agar. In no case were contaminating bacteria detected, indicating that the level of contamination was not significant.

Effect of Surface Plating upon Bacterial Count. Up to this point, all bacterial counts had been made using the pour plate technique. However, several

reports in the literature suggested that higher counts were obtained with a surface plating method.

The agar plates for the surface plating were poured several days prior to the experiment and were stored in the 28°C incubator. This permitted adequate drying for an inoculum no larger than 0.5 ml. In the inoculation of a plate, the selected volume of the 10^{-6} dilution was pipetted onto the surface of the agar and distributed as uniformly as possible over the surface with a sterile glass rod.

Each of the following culture media was inoculated by the pour plate and surface plate techniques:

1. MSAA, with SS-ASML diluent
2. MSAA, with tap water diluent
3. MSCA, with SS-ASML and 300 mg/l sodium caproate
4. MSCA, with tap water and 300 mg/l sodium caproate
5. NA-SS-ASML

The results in Table 13 show no definite advantage of one method over the other in regard to productivity. However, since replica plating was to be used in future experiments, surface plating was adopted as standard procedure.

Effect of Aeration Tank Liquor in Synthetic Medium upon Growth of Acetate-Utilizers. It was possible that the synthetic MSAA might lack some essential growth factor which might be available to the plant activated sludge bacteria in the mixed liquor; therefore, catalytic amounts of ASML might increase productivity.

Each of the following culture media - NA, MSA, and MSAA - were prepared using each of the following diluents: tap water, 1% ASML in tap water and 5% ASML in tap water. The ASML was prepared as follows: a sample of ASML from the Metropolitan Nashville Sewage Treatment Plant was allowed to settle and 500 mg of supernatant liquid was filtered through cotton which has previously been washed with

distilled water.

Activated sludge mixed liquor from the Metro Plant was used as source of organisms. The results are shown in Table 14. The presence of ASML in these amounts appeared to reduce the productivity of NA below that given by NA-tap water, while exerting no apparent effect upon the growth of acetate-utilizers on the synthetic medium.

Enumeration of Bacteria in Activated Sludge Mixed Liquor Capable of Utilizing Individual Saturated Monocarboxylic Acids as Sole Carbon Source. Part I. A 100-ml sample of ASML from the Franklin Plant (i.e., a suspension of unwashed activated sludge solids (MLSS) unadjusted in concentration) was blended 135 sec and serial dilutions were prepared in sterile BOD dilution water (pH 7.1 - 7.2).

Bacterial plate counts were set up using the following culture media, all prepared with SS-ASML as diluent:

1. NA, for total count
2. MSA
3. MSA - formate agar, i.e., MSA containing 300 mg/l sodium formate.

4 to 13. MSA - fatty acid agars, i.e., containing 300 mg/l of sodium salts of acetic, propionic, butyric, valeric, caproic, caprylic, lauric myristic, palmitic and stearic acid, respectively.

The surface plating method was used. Each of six plates of NA-SS-ASML was inoculated with 0.30 ml of 10^{-6} dilution of MLSS. Each of two plates of the other media was inoculated with 0.1 ml of the 10^{-4} dilution. All plates were incubated at 25°C. for 4 days when counts were made. Results are shown in the second column of Table 15.

Part II. One of the NA-SS-ASML plates showing between 50-100 colonies (in this case, 65) was chosen for use as Master Template for replica plating, i. e.,

this plate was used to impress the pattern of colony dispersion on a sterile velveteen cloth. This cloth was then used to inoculate one plate each of the culture media used in Part I. The last plate in the replica plating series was a second NA-SS-ASML plate which served as a check for possible exhaustion of the imprinting cloth. The fact that the colony count of the second NA-SS-ASML plate was the same as the initial NA-SS-ASML plate indicated that a single imprint on the velveteen cloth deposited enough organisms to inoculate all the plates in the series.

The number of colonies on the Master Template capable of growing on the various fatty acid media are shown in column 4 of Table 15. The increased proportion of the total population (65) growing on the synthetic media indicates that preliminary growth on NA-SS-ASML either (a) acclimated many ASML bacteria to growth on the various fatty acids as carbon source, or (b) simply strengthened the organisms, making them better able to make the transition to synthetic media, or (c) acclimated the organisms to growth on the MSA medium.

E. Analysis of Primary Effluent for Content of Satureated Monocarboxylic Acids.

Based upon recognized ecological principles, it would be expected that the ability of activated sludge to oxidize fatty acids during the first few hours of exposure would be the consequence of the same fatty acids in the feed entering the aeration tank, e.g., in the primary effluent.

Preliminary chemical studies were directed to the development of a method for the quantitative recovery of the acids from the primary effluent. Initial work was done on the lower fatty acids up to and including valeric (pentanoic) acid.

Concentration. The extremely low concentration of these acids expected in the primary effluent, e.g., in the microgram per liter range, meant that concentration must be carried out. Freeze concentration appeared to be the method of choice, since this technique permits concentration of dissolved solids, while holding loss from volatility and bacterial action at a minimum.

Initial work was based upon the methodology of Murtaugh and Bunch (Jour. WPCF, 33:122, 1961), i.e., ten-liter samples were concentrated using the principle of the Virtis 3-100 freeze-concentration. In this apparatus, freezing proceeds from the bottom of the vessel upwards, while the solids (theoretically) remain dissolved in the unfrozen liquid. However, it seemed to the present investigators that it would be impossible to prevent precipitation and settling of solids, even with stirring, and that some solids would inevitably be incorporated into and discarded with the ice. It was felt that this might be an important source of error.

Murtaugh and Bunch attempted to reduce coagulation and settling during freezing by prior passage of the sample through a cation exchanger to remove calcium and magnesium. However, this ion-exchange treatment could not fail to adsorb some of the organic constituents of the primary effluent. This was another source of error. Also, it was doubted that removal of calcium and magnesium ions could prevent entirely the precipitation of solids resulting from the physical phenomenon of concentration.

An alternate method of freeze concentration was studied. A 10-liter volume of primary effluent was poured into several metal buckets which were placed in a food freezer at -15 to -22°C. After 6-8 hr, a bucket was removed from the freezer, two holes were made in the surface ice, and the unfrozen liquid in the center poured into another metal bucket. The ice cake was transferred to a plastic

dishpan.

The ice cake showed three zones; (a) dirty bottom ice containing sediment, (b) clean ice making up the top 3 or 4 inches, and (c) an intermediate zone of variable clarity. The clean ice was separated by careful cracking, the interior surface rinsed with distilled water, and the ice was discarded. The rinse water was added to the unfrozen liquid for further processing.

The dirty ice and the intermediate ice were returned to the freezing vessel and were melted by placing the vessel in hot water. The dishpan was rinsed with distilled water and the rinse water returned to the freezing vessel. The latter was then placed in the food freezer for another freeze cycle.

Freezing was continued in this fashion until the volume of dirty ice in the bottom plus the volume of unfrozen liquid totaled about 1.5 liters. This required four or five freezing cycles.

Further concentration in a flash evaporator at 45°C. was studied. This was very time-consuming. Also, there was some concern about possible loss of the more volatile fatty acids during the process.

Extraction. This phase of the study has proved very difficult. The concentrate (i.e., the liquid phase plus sedimented solids) was transferred quantitatively to a 2000-ml separatory funnel and extracted with organic solvent in two stages. In some experiments 20% sodium chloride was dissolved in the aqueous phase to reduce the solubility of solvent in the water.

The solvent was used in a ratio of 75 ml per liter of concentrate. The solvent-concentrate mixture was shaken for 2 min at a rate sufficient to get good mixing, but not so violently as to produce a permanent emulsion at the interface. The mixture was allowed to stand two minutes, then was shaken again. The mixture was allowed to stand two minutes for phase separation. The bottom layer was removed for further processing or discarding depending upon the solvent

used.

The first extraction of the concentrate was carried out at alkaline pH. The aqueous phase was adjusted to about pH 10.5 with NaOH solution. This converted all acids to the salt form. The concentrate was extracted five times with 75-ml volumes of organic solvent in the manner described above. The solvent in this case was discarded, since extraction under these conditions removes lipid materials other than the fatty acid salts which remain in the aqueous phase.

The water solution was then adjusted to pH 3.5 with H_2SO_4 solution. Five extractions with 75-ml volumes of solvent were then performed. In this case the solvent is retained since the fatty acids are now in the solvent phase.

Initial experiments used chloroform as extracting solvent but recoveries were very poor. Later experiments employed ether as solvent.

The combined volumes of ether used in the second stage extraction were transferred to the flash evaporator. Using partial vacuum and water bath at 42°C, the volume was reduced to about 15 ml. This volume was transferred by pipette to a 50-75 ml evaporating flask. The large evaporating flask was rinsed with several 3-ml volumes of ether which were combined with the original 15 ml. The combined volume was then concentrated to about 6 ml. Anhydrous Na_2SO_4 was added directly to the contents of the flask. After overnight contact it was assumed that all water had been absorbed by the Na_2SO_4. The liquid was transferred to a test tube and the flask rinsed several times with small volumes of anhydrous ether.

Since it was desired to have a final volume of 2.0 ml for gas chromatography, further concentration was necessary. A piece of glass tubing with a capillary tip connected to an air outlet by way of a filter was inserted above the surface of the solution. A gentle flow of air was sufficient to evaporate the ether to

the 2.0-ml mark on the test tube or cylinder. The solution was carefully swirled over the inner surface of the test tube to dissolve any dried residue. The solution of fatty acids was now ready for the GC work.

Gas chromatography. The 2.0 ml solution was analyzed for content of fatty acid in the Beckman GC 4 using the following experimental conditions:

Column - neopentyl glycol adipate on Chromsorb W, acid-treated

Detector - flame ionization

Carrier gas - helium

Standards - 0.01 and 0.05% of chromato-quality acids (99+% pure, from Applied Science Laboratories) in ethyl ether, chloroform or water, depending upon solvent employed.

The analytical procedure was alternate injection of equal volumes (2 to 10µl) of sample and standard solutions with all experimental conditions maintained constant. The peaks on the sample run were identified by comparison with the retention times of the components of the standard solution. The concentration of acids was determined from peak areas measured by the planimeter method.

Variables investigated. In order to develop a reliable method, i.e., one which would measure accurately the concentrations of fatty acids in the primary effluent, the efficiency of the various steps in terms of percentage recovery of acids, as well as the effect of variation in the steps had to be studied. The following variations were employed:

Original sample

(a) 10 liters primary effluent

(b) 10 liters P.E. plus known amounts of fatty acid salts to give concentrations in the range expected in the P.E.

Extraction step

(a) Extraction of standard salt solution which had been adjusted to the acid pH.

(b) Usual two series of extractions of standard salt solution, i.e., to alkaline pH, then to acid pH.

(c) Extraction of standard salt solution adjusted to acid pH and 2% NaCl added.

(d) Same as (c) but with doubled volume of solvent used.

(e) Same as (c) and (d) but with 20% NaCl added.

(f) Extraction of standard salt solution on acid side but using hexane as solvent.

(g) Elimination of acid extraction step by going directly from alkaline solution with phosphoric acid and analyzing the water solution on the GC.

Results. Except in the case of (g), the results obtained were uniformly poor, i.e., the percentage recovery of added fatty acids was very low - ranging from 3 to 19%.

Future work. Intensive efforts are being made to locate the step or steps causing the loss of fatty acids.

TABLE 1 - EFFECT OF BLENDING TIME UPON BACTERIAL COUNT OF ACTIVATED SLUDGE

Expt.	SPC/ml for Blending Times, sec.					
	90	105	120	135	150	180
1	40[a]		44		20	
2	100		70		100	80
3	70		210		90	130

[a]All values in table x10^6

TABLE 2 - EFFECT OF INCUBATION PERIOD UPON BACTERIAL COUNT OF ACTIVATED SLUDGE

Expt.	SPC/ml for Incubation Time, days					
	3	4	6	8	11	14
1	90[a]	130	200	250	270	
2	60	100	160	250	280	
3			240	304		342

[a]All values in table x10^6

TABLE 3 - EFFECT OF PRIMARY EFFLUENT IN CULTURE MEDIUM UPON BACTERIAL COUNT

Medium	SPC/ml @ 6 days
NA + dist	240[a]
NA + PE	70
NA + filt PE	74

[a]All values in table x 10^6

TABLE 4 - EFFECT OF AERATION TANK MIXED LIQUOR IN CULTURE MEDIUM UPON BACTERIAL COUNT

Medium	SPC/ml @ 6 days	
	Expt. 1	Expt. 2
NA + dist	32[a]	204
NA + ASML	44	106
NA + filt ASML	16	92

[a]Values x 10^6

TABLE 5 - EFFECT OF ENRICHED CULTURE MEDIA UPON BACTERIAL GROWTH

Medium	SPC/ml @ 4 days	
	Expt. 1	Expt. 2
NA - tap	135[a]	155
TSA - tap	26	30
DSTA - tap	15	9

[a]Values x 10^6

TABLE 6 - EFFECT OF MIXTURE OF PRIMARY EFFLUENT AND AERATION TANK MIXED LIQUOR UPON BACTERIAL GROWTH

Medium	SPC/ml @ 4 days	
	Expt. 1	Expt. 2
NA - tap	135[a]	155
NA - SS - ASML	130	168
TSA - tap	26	30
TSA - SS - ASML	27	29
DSTA - tap	15	9
DSTA - SS - ASML	15	15

[a]Values x 10^6

TABLE 7 - BACTERIAL COUNT IN PRESENCE OF FATTY ACID AS SOLE CARBON SOURCE

Medium	SPC/ml	
	2 days	4 days
NA	5,000,000	100,000,000
MSA	<10,000	600,000
MSAA	1,350,000	3,300,000
Ratio NA/MSAA	3.7 to 1	30 to 1

TABLE 8 - BACTERIAL COUNT IN PRESENCE OF FATTY ACID AS SOLE CARBON SOURCE

Medium	SPC/ml	
	2 days	4 days
NA	6,000,000	90,000,000
MSA	<10,000	710,000
MSAA	1,700,000	3,440,000
Ratio NA/MSAA	3.5 to 1	27 to 1

TABLE 9 - PROPORTION OF ACETATE-UTILIZERS IN TOTAL POPULATION WHEN BOD DILUTION WATER WAS USED IN DILUTION BLANKS

Medium	SPC/ml	
	2 days	4 days
NA	75,000,000	158,000,000
MSA	70,000	190,000
MSAA	1,810,000	4,700,000
Ratio NA/MSAA[a]	43 to 1	35 to 1

[a]MSAA corrected for MSA count

TABLE 10 - EFFECT OF BEEF EXTRACT UPON GROWTH OF ACETATE-UTILIZERS IN SYNTHETIC MEDIUM

Medium	SPC/ml	
	2 days	4 days
NA	20,000,000	99,000,000
MSA	50,000	470,000
MSAA	700,000	3,250,000
MSA + BE	70,000	580,000
MSAA + BE	720,000	3,000,000
Ratio NA/MSAA	31 to 1	36 to 1
Ratio NA/MSAA-BE	31 to 1	41 to 1

TABLE 11 - EFFECT OF VARIATION IN PHOSPHATE BUFFER SYSTEM ON BACTERIAL COUNT USING SYNTHETIC MEDIUM

Medium	SPC/ml	
	2 days	4 days
NA	10,000,000	53,000,000
MSA	40,000	300,000
MSAA	300,000	1,230,000
Ratio NA/MSAA	38 to 1	57 to 1
MSA - mod	<10,000	240,000
MSAA - mod	730,000	3,890,000
Ratio NA/MSAA - mod	14 to 1	15 to 1

TABLE 12 - EFFECT OF METHOD OF STERILIZATION OF SYNTHETIC MEDIUM UPON PRODUCTIVITY

Medium	SPC/ml	
	2 days	4 days
MSAA	1.3[a]	40
MSAA - auto	0.9	12.8
MSPA	3.7	70
MSPA - auto	1.0	42

[a]Values x 10^6

TABLE 13 - COMPARATIVE BACTERIAL COUNTS BY POUR PLATE AND SURFACE PLATE TECHNIQUES

Medium	@ 2 days	
	SPC/ml	SurPC/ml
MSAA - SS - ASML	11[a]	11
MSAA - tap	9	20
MSCA - SS - ASML	1	2.5
MSCA - tap	7	3
NA - SS - ASML	56	58
	@ 4 days	
	SPC/ml	SurPC/ml
MSAA - SS - ASML	25	27
MSAA - tap	14	58
MSCA - SS - ASML	3.2	12
MSCA - tap	11	5
NA - SS - ASML	158	138

[a]Values x 10^6

TABLE 14 - EFFECT OF AERATION TANK MIXED LIQUOR IN SYNTHETIC MEDIUM UPON GROWTH OF ACETATE-UTILIZERS

Medium	SurPC/ml	
	2 days	4 days
NA - tap	33[a]	210
NA - 1% ASML	20	120
NA - 5% ASML	20	120
MSA - tap	0.8	4.8
MSA - 1% ASML	1.0	2.8
MSA - 5% ASML	1.0	5.0
MSAA - tap	1.3	9.9
MSAA - 1% ASML	1.6	9.6
MSAA - 5% ASML	1.2	9.7

[a]Values x 10^6

TABLE 15 - BACTERIA IN ACTIVATED SLUDGE MIXED LIQUOR CAPABLE OF UTILIZING SATURATED MONOCARBOXYLIC ACIDS

Medium	Part I SPC/ml	Part I Ratio NA : Fatty Acid Medium[a]	Part II Colonies Replicated
NA	227[b]	-	65
MSA	4.6	-	45
Formate	14.7	22:1	38
Acetate	15.6	25:1	38
Propionate	18.8	16:1	47
Butyrate	14.9	22:1	37
Valerate	24.3	12:1	36
Caproate	14.7	22:1	41
Caprylate	15.9	20:1	36
Laurate	15.9	20:1	55
Myristate	6.9	99:1	42
Palmitate	0.75	-	45
Stearate	12.0	30:1	22
NA	-	-	65

a Count on fatty acid medium corrected for MSA count.

b Values in this column x 10^6